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THE ROLE OF DIFFERENT INTERCROPS IN GUAVA ORCHARDS FOR DIEBACK DISEASE DEVELOPMENT AND INOCULUM BUILDUP IN PAKISTAN

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Abstract
Guava (Pasidium guajava Linn.) belongs to the family Myrtaceae, it is an important member of this family. Guava dieback caused by Colletotrichum gloeosporioides, Surveys were conducted in different guava growing areas of Pakistan for the data documentation of different intercrops which play role in disease development. Eight different intercrops were observed in guava orchards and disease prevalence was high in those orchards were mango and citrus were cultivated as compared to other orchards, after the samples processing and identification the Colletotrichum gloeosporioides was isolated only from mango and citrus intercrops samples. The pathogenicity test was performed by following Koch's postulates for the confirmation of pathogen and finding significant results, Colletotrichum gloeosporioides cause the successful infection on guava healthy plants but the virulence of mango isolates was high as compared to citrus isolates. After the confirmation of pathogenicity results the Colletotrichum gloeosporioides (that were isolated from guava disease samples and intercrops) were sequenced and find the significant results, these are the same isolates, which means that mango and citrus plants as intercrop in guava orchard play role in disease development that's why disease prevalence was high in those orchards.
Keywords: guava, intercrops, disease prevalence, dieback, pathogenicity

INTRODUCTION

The Agri-Horti system is a cropping system in which different crops like (fruit trees, ornamental trees, or vegetable crops) cultivate at the same place for maximum utilization of natural resources and remarkably increases the return per unit area per unit time (Gill and Bisaria, 1995). Intercropping is a good technique of land utilization for optimum production (Bhatnagar et al., 2007).

Intercropping in orchards is a common practice in many countries (Ouma and Jeruto, 2010). Suitable Intercrops improve fruit production of the orchard as compared to the non-intercropped orchards. Such crops help increase the crop yield by fixing nitrogen biologically in the soil (Aziz et al., 2008; Srivastava et al., 2007). Furthermore, intercrops suppress the weeds and improve the orchard's yield (Linares et al., 2008). The leguminous intercrops are the most effective crop because of their desirable impact on improving the nutrient status of the soil and fruit plant of the orchard, and yield stability

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are more significant with intercropping as compared to sole cropping. However, the success of intercropping systems depends mostly on selecting suitable intercrop and implementation of an appropriate intercropping systems that can provide considerable yield advantages as compared with the sole cropping without exhaustion of soil and orchard health (Swain, 2016; Din et al., 2012). Similarly, vegetable crops specially tuber crops like Suran, Turmeric, Arvi, and Bunda are suitable as intercrop in guava orchards due to their shade-loving nature even in the old dense shade of guava plants (Singh, et al., 2014).

Besides the beneficial aspects of the intercropping system there are several challenges also like the erroneous choice of intercrops desperately affect the orchard health and ultimately yield. For example, the cultivation of berseem in Citrus orchards affects adversely the yield of citrus because both crops have different input requirements. The Berseem requires irrigation weekly while Guava requires less water, and excessive use of water deoxygenates the root system of orchard plants (Ijaz et al., 2014). Similarly, at the harvesting time of wheat, irrigation is stopped, but orchards require irrigation, which adversely affects yield and growth (Srivastava et al., 2007; Sarwar et al., 2012). A similar pattern of intercropping is very common in Guava orchards of Pakistan, where the farmers grow fodders, vegetables, and field crops in Guava orchards (Unpublished data/ Personal observation). *Colletotrichum gloeosporioides* is an extensively distributed and common plant pathogen in the world (Sutton,1992; Cannon *et al*., 2008) which has a wide host range and it infects about 470 different plant host species and some important economic crops such as avocado, mango, beans, citrus plant, cotton, soybean, tomato, wheat, cucurbit, cereals, and legumes (Sharma and Kulshrestha, 2015).

The die-back disease prevailed in the Guava orchards of Pakistan with varying intensities depending upon the choice of variety, regions, horticultural and filed sanitation practices. This research plan was designed to screen the variety of intercrops commonly grown in guava orchards that are likely serving as an alternate host that contributes to the buildup of the inoculum levels and determining if any of the intercrops are on lists of hosts of *Colletotrichum spp*. that might be the same as those on Guava. Samples comprising of aerial plant parts of intercrops from fields that have a high incidence of Guava dieback were examined to determine the association of *Colletotrichum* spp. (Safdar *et al.,* 2015). The role of intercrops as alternate hosts of the die-back pathogen was determined based on the frequency of *Colletotrichum* spp. associated with collected samples and testing the Koch's postulates with selected *C. gloeosporioides* isolates. The second objective of this study was to find out the intercrops that are suitable having compatibility with Guava. The results obtained were translated into advisory to the Guava growers upon the appropriate choice of crops for intercropping with Guava. This research was one of the cultural control a part of Integrated management of Guava die-back under the CAS Punjab Agricultural Research Board funded project #954.

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Material and methods

Survey

Surveys were conducted to different Pakistan areas where guava was cultivated with other intercrops for data documentation of Intercrops, which play a role as alternate host in disease development. Disease incidence and severity were recorded from those areas. Survey areas and cropping pattern of those areas were given below in table 3.1:

Table 3.1: Different survey areas and cropping system

	Sr No.	Districts	Location & GPS Coordinates	Site	Plant age	Variety	Cropping system
PUNJAB	1.	**Faisalabad**	UAF 31.4303, 73.0672	Orchard 1	5 Year	Sorahie, Gola	Sole cropping
				Orchard 2	10 Year	Sorahie, Gola	
			Pars 31.3630, 72.9876	Orchard 1	15 Year	Sorahie, Gola	Intercropping
			Samundri 31.0646, 72.9520	Orchard 1	8 Year	Sorahie, Gola	Sole cropping
				Orchard 2	12 Year	Sorahie, Gola	
			Jaranwala 31.3454, 73.4298	Orchard 1	5 Year	Sorahie, Gola	
				Orchard 2	15 Year	Sorahie, Gola	
				Orchard 3	10 Year	Sorahie, Gola	
			Tandlianwala 31.0368, 73.1379	Orchard 1	8 Year	Sorahie, Gola	Intercropping
				Orchard 2	5 Year	Sorahie, Gola	Sole cropping
	2.	**Bahawalpur**	IUB 29.3544, 71.6911	Orchard 1	5 Year	Sorahie, Gola	
			Lal Suhanra 29°19, 71°55	Orchard 1	3 Year	Sorahie, Gola	
				Orchard 2	7 Year	Sorahie, Gola	
	3.	Bahawalnagar	Bahawalnagar 30.0025, 73.2412	Orchard 1	17 Year	Sorahie, Gola	Intercropping
				Orchard 2	3 Year	Sorahie, Gola	
			Fort Abbas 29.1931, 72.8575	Orchard 1	2 Year	Gola	Sole cropping
				Orchard 2	13 Year	Gola	Intercropping
				Orchard 3	4 Year	Sorahie, Gola	Sole cropping
			Dolat pur 32° 41, 73° 18	Orchard 1	20 Year	Sorahie, Gola	
				Orchard 2	14 Year	Sorahie, Gola	
				Orchard 3	5 Year	Sorahie, Gola	
			Chishtian 29.7956, 72.8634	Orchard 1	8 Year	Sorahie, Gola	
				Orchard 2	12 Year	Sorahie, Gola	
	4.	Layyah		Orchard 1	20 Year	Gola	Intercropping

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			Layyah 30.9693, 70.9428	Orchard 2	10 Year	Gola	
			Chowk Azam 30.9647, 71.2043	Orchard 1	10 Year	Sorahie, Gola	
	5.	Hafizabad	Hafizabad 32.0712, 73.6895	Orchard 1	7 Year	Sorahie, Gola	
				Orchard 2	12 Year	Sorahie, Gola	
	6.	Kasur	Pattoki 31.0249, 73.8479	Orchard 1	10 Year	Sorahie, Gola	Sole cropping
				Orchard 2	15 Year	Sorahie, Gola	
	7.	Nankana Sahib	Shah kot 31.5757, 73.4815	Orchard 1	9 Year	Sorahie, Gola	
				Orchard 2	4 Year	Sorahie, Gola	
				Orchard 3	3 Year	Sorahie, Gola	
	8.	Sheikhupura	Sharak Pur 31°27'28.8, 74°06'00.0	Orchard 1	12 Year	Sorahie, Gola	
				Orchard 2	20 Year	Sorahie, Gola	
	9.	Multan	Multan 30.1575, 71.5249	Orchard 1	5 Year	Sorahie, Gola	
				Orchard 2	10 Year	Sorahie, Gola	
	10.	Vehari	Vehari	Orchard 1	12 Year	Sorahie, Gola	
			Burewala 30.1593, 72.6943	Orchard 1	8 Year	Sorahie, Gola	
				Orchard 2	10 Year	Sorahie, Gola	
SINDH	1.	Banbhore	Thatta 24.7475, 67.9106	Orchard 1	10 Year	Sorahie, Gola	
				Orchard 2	35 Year	Sorahie, Gola	
				Orchard 3	13 Year	Sorahie, Gola	
	2.	Hyderabad	Hyderabad 25.3960, 68.3578	Orchard 1	25 Year	Sorahie, Gola	
				Orchard 2	10 Year	Sorahie, Gola	Intercropping
				Orchard 3	17 Year	Sorahie, Gola	
				Orchard 4	8 Year	Sorahie, Gola	
			Tando Jahanian 25.3924, 68.3507	Orchard 1	12 Year	Sorahie, Gola	
				Orchard 2	15 Year	Sorahie, Gola	
				Orchard 3	7 Year	Sorahie, Gola	Sole cropping
	3.	Shaheed Benazir Abad	Naushahro Feroze 26.8463, 68.1253	Orchard 1	13 Year	Sorahie, Gola	
				Orchard 2	15 Year	Sorahie, Gola	
				Orchard 3	9 Year	Sorahie, Gola	
				Orchard4	10 Year	Sorahie, Gola	
	4.	Larkana	Larkana 27.5570, 68.2028	Orchard 1	15 Year	Sorahie, Gola	Intercropping
				Orchard 2	17 Year	Sorahie, Gola	
				Orchard 3	10 Year	Sorahie, Gola	Sole cropping
KPK	1.	Hazara	Hazara 32.7962, 74.2840	Orchard 1	12 Year	Sorahie, Gola	
				Orchard 2	5 Year	Sorahie, Gola	Intercropping
			Haripur 33.9946, 72.9106	Orchard 1	15 Year	Sorahie, Gola	
				Orchard 2	8 Year	Sorahie, Gola	
	2.	Mardan		Orchard 1	10 Year	Sorahie, Gola	

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			Mardan 34.1989, 72.0231	Orchard 2	12 Year	Sorahie, Gola	
	3.	Kohat	Kohat 33.5889, 71.4429	Orchard 1	7 Year	Sorahie, Gola	
				Orchard 2	15 Year	Sorahie, Gola	
				Orchard 3	20 Year	Sorahie, Gola	Intercropping

Screening of Intercrops to determine their role as alternate hosts of *Colletotrichum spp.*

Screening of intercrops was done by first determining the intercrops are on lists of hosts of *Colletotrichum spp*. that might be the same as those on Guava. It was also determined if a variety of intercrops commonly grown in guava orchards are likely to serve as an alternate host contributing to the inoculum levels' buildup. The role of intercrops as alternate hosts of the die-back pathogen was determined based on the frequency of *Colletotrichum* spp. associated with collected samples, identification on a morphological basis, and then pathogenicity assay with *Colletotrichum gloeosporioides.*

Sampling and processing (Isolation, Purification, and identification of pathogen).

Sampling was done from Guava and intercrops by observing the symptoms on different parts of the plants. Branches, leaves, roots, and fruit samples were collected from Guava and intercrops to check intercrop's role in disease development. Both diseased and healthy samples were collected from the intercrops to confirm the association of pathogens, which play a role in disease development. These collected samples were brought into the fungal molecular biology lab (FMB) and further processed, like isolation, purification, and pathogen identification. After isolation, *Colletotrichum* species were identified on a morphological basis, and find the frequency of *Colletotrichum* species on each intercrop.

Pathogenicity Assay

The *Colletotrichum gloeosporioides* isolated from different intercrops were selected for the pathogenicity test by following the Koch postulates (Agrios 2005) to find out that was play a role in disease development in Guava or not. Four *Colletotrichum gloeosporioides* isolates isolated from Mango and Citrus at different locations were selected, and spore suspension was prepared from 7 days old culture. To prepare the inoculum, 10mL of sterilized water was added into a pure culture Petri plate, and a microscope glass slide slightly scraped the surface. The suspension was then filtered via two covers of muslin cloth, and the conidia concentration was examined under the microscope and set the 10^6 spores/mL using the hemocytometer (Oo *et al.,* 2018). Suspension of each isolate was sprayed on healthy guava plants in the greenhouse, and data was recorded after four weeks of inoculation by using the disease rating scale that was modified by following the other scientists rating scales against die-back disease (Ramos *et al.,* 1997, Mayee and Datar 1986, Inglis *et al.,* 1988, Wangungu *et al.,* 2011). The infection rate of each isolate was calculated by following the McKinney (1923) index.

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Molecular evaluation

After the confirmation of pathogenicity assay, these isolates were selected for sequencing to find out whether these are the identical isolates of *Colletotrichum gloeosporioides* that were isolated from guava plants disease samples or not.

Results

Comparison of survey intercrops in guava orchards with Literature

The literature on guava intercrops was studied; to know which crops were grown commonly in Pakistan and worldwide. Different intercrops like Sorghum, Alfalfa, Berseem, Maize, Wheat, mango, and citrus were surveyed in Pakistan. The complete detail of intercrops was given below in table 4.1:

Table 4.1: Cultivation of guava intercrops worldwide and Pakistan

The Literature of Guava intercrops (Worldwide)			**Survey intercrops (Pakistan)**				
Maincrop	Intercrop	Reference		Sr. No.	Districts/Location	Orchard type (multiple cropping)	
						Summer	**Winter**
Guava	Coconut	Manna and Singh, 2000					
	Guar or cluster bean (Cyamopsis tetragonolobu	(Shweta et al., 2015)		1	Fort Abbas (2nd Orchard)	Sorghum	Alfalfa
	Mungbean (Vigna radiata L.)			2	Bahawalnagar (1st Orchard)	Sorghum	Berseem
	Cow pea (Vigna unguiculata		**PUNJAB**	3	Bahawalnagar (2nd Orchard)	Maize	Wheat
	Arvi (Colocasia esculenta var. antiquerum),	(Singh et al., 2016)		4	Layyah (1st Orchard)	Sorghum	Alfalfa
	Bunda (Colocasia esculenta var. esculenta),			5.	Layyah (2nd Orchard)	Mango	Mango
	Suran (Amorphophallus companulatus L.)			6.	Chowk Azam (1st Orchard)	Citrus	Citrus
	Turmeric (Curcuma domestica),			7.	PARS (1st Orchard)	Mango	Mango
	Banana (Musa acuminaia)	Ghosh et al., 2017		8.	Tandly wala (1st Orchard)	Glad	Glad
	Eggplant (Solanum melongena)		**SINDH**	1	Hyderabad (2nd Orchard)	Alfalfa	Wheat

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	pigeon pea (Cajanus cajan), Paddy (Oryza sativa)	Raut, 2018		2	Larkana (1st Orchard)	Alfalfa	Berseem
	Black gram (Vigna mungo)		KPK	1	Hazara (2nd Orchard)	Sorghum	Wheat
	Ginger (Zingiber officinale)			2	Kohat (3rd Orchard)	Alfalfa	Wheat

Host rang of *Colletotrichum* spp.

Colletotrichum is a broad host range species. It caused many diseases in different host plants. The literature was reviewed to find the host range of *Colletotrichum* species and which host crops were grown commonly in guava orchards. The comparison of the host range of *Colletotrichum* species worldwide and in Pakistan is given below in table 4.2:

Table 4.2: Host range of *Colletotrichum* species and comparison of survey intercrops

The literature of Colletotrichum host species (Worldwide)				**Survey host crops (Pakistan)**
Disease/host	**Host**	**Reported**	**References**	
Bitter rot	Apple	North Carolina	(Shane and Sutton, 1981).	**1: Sorghum** Common name: Jowar English name: Sorghum Scientific Name: Sorghum bicolor
Fruit rot	Apple, pear	USA	(Sutton 1990).	
Anthracnose	Avocado	Australia Israel, South Africa Sri Lanka	(Fitzell, 1987), (Binyamini and Nadel, 1972) Darvas and Kotze, 1987) (Sivanathan and Adikaram, 1989)	**2: Alfalfa** Common name: Lucerne English name: Alfalfa Scientific Name: Medicago sativa
Anthracnose	Almond, avocado	Israel	(Binyamini and Nadel, 1972; Shabi and Katan, 1983)	
Anthracnose	Avocado	Australia, South Africa	(Giblin and Coates, 2007)	
Anthracnose	**Citrus**	Belize	(Fagan, 1980	
Anthracnose	Dragon fruit	Peninsular Malaysia	(Masyahit et al., 2009).	
Anthracnose	Trichosanthes kirilowii	China	(Li and Zhang, 2007).	
Anthracnose	Lupins	Western Australia	(www.hannafords.com).	
Anthracnose	Mango	first reported from Puerto Rico later from Hawaii Florida, Cuba, Philippines, Columbia, South Africa, Brazil,	(Ploetz and Prakash, 1997 (Collins, 1903) (Higgins, 1906), (Fawcett, 1907), (Cardin, 1910), (Wester, 1911), (Taro, 1929), (Doidge, 1932),	**3: Maize** Common name: Makai English name: Maize Scientific Name: Zea mays

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		The United States and Pakistan	(Bitancounrt, 1938), (Traub and Robinson, 1938) (Sattar and Malik, 1939)	
Anthracnose	**Mango**	South Asia	(Dodd et al., 1991).	
Anthracnose	Coffee berries	Vietnam	(Nguyen et al., 2009)	
Anthracnose	Olive	southern Montenegrin coast near Ulcinj	(Latinovic and Vucinic, 2002)	
Anthracnose	Onion	Brazil	(Barbosa, 2001).	
Anthracnose	Papaya fruits	Hawaii	(Dickman and Alvarez, 1938).	
Anthracnose	Red pepper		(Lee and Chung, 1995).	
Anthracnose	Pepper	hot, humid tropics of Asia.	(Manandhar et al., 1995	
Anthracnose	Pepper		Park and Kim	
Anthracnose	Bell pepper	Himachal Pradesh, India.	(Gupta et al., 2009).	
crown rot	Strawberry		(Freeman and Katan, 1997; Howard and Albregts, 1984; Howard et al., 1992)	
Anthracnose	Tulip tree	Korea	(Choi et al., 2012)	
Die-back/ Anthracnose	Yam Water yam	Africa, Central South America, parts of Asia, the Caribbean and Pacific islands	(Coursey, 1967; Adelusi and Lawanson, 1987).	
Anthracnose	**Maize**	Texas, USA Kentucky, USA China	Sukno et. al., 2008. Torres, 2013 Duan et. al., 2019	
Anthracnose	**Sorghum**	Puerto Rico, USA Texas, USA Andhra Pradesh, India Spain USA USA	Erpelding, 2010 Cardwell, 1989 Mathur et. al., 1997 Baroncelli et. al., 2014 Crouch, and Beirn, (2009)	
Anthracnose	**Alfalfa**	Wisconsin, USA Serbia	Samac et. al.,2014 Vasić et. al., 2014	

4: Wheat
Common name: Gandum
English name: Wheat
Scientific Name: Triticum

5: Mango
Common name: Mango
English name: Mango
Scientific Name: Mangifera indica

6: Citrus
Common name: Kinnow
English name: Citrus
Scientific Name: Citrus × latifolia

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		Uttarakhand (India)	B. Kumar and K.P. Singh, 2018	
Anthracnose	**Wheat**	USA NARC, Islamabad, Pakistan. India	Crouch, and Beirn, (2009) Iftikhar et. al., 2008 Sharma and S. Kulshrestha, 2015	

Disease Prevalence of different intercropped guava orchards at different locations

Data was recorded from guava orchards where in summer and winter season different intercrops were cultivated. The disease incidence, severity, environmental conditions (temperature and rainfall), varieties and orchard age were recorded from those areas, which is given detailed in below table 4.3:

Table 4.3: Disease incidence and severity of guava orchards at different locations.

	Sr. No.	Districts/Location Coordinates	Orchard type (multiple cropping)		Orchard Age (Years)	Varieties	Disease prevalence (Means ± S. E)		Temp & Rainfall
			Summer	**Winter**			Severity	Incidence	
PUNJAB	1	Fort Abbas 29.1931, 72.8575 (2nd Orchard)	Sorghum	Alfalfa	13	Gola	36 ± 0.53	50 ± 2.90	35° 37 mm
	2	Bahawalnagar 30.0025, 73.2412 (1st Orchard)	Sorghum	Berseem	17	Sorahie	33 ± 0.47	55 ± 2.10	33° 50 mm
						Gola	35 ± 0.57	58 ± 1.76	
	3	Bahawalnagar 30.0025, 73.2412 (2nd Orchard)	Maize	Wheat	3	Sorahie	13 ± 1.85	15 ± 2.60	
						Gola	16 ± 0.57	17 ± 1.76	
	4	Layyah 30.9693, 70.9428 (1st Orchard)	Sorghum	Alfalfa	20	Gola	35 ± 0.37	50 ± 2.90	16° 74 mm
	5.	Layyah 30.9693, 70.9428 (2nd Orchard)	Mango	Mango	10	Gola	42 ± 0.57	65 ± 2.90	
	6.		Citrus	Citrus	10	Sorahie	39 ± 0.88	60 ± 2.15	31°

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		Chowk Azam 30.9647, 71.2043 (1st Orchard)				Gola	41 ± 1.20	62 ± 2.30	201 mm
	7.	PARS 31.3630, 72.9876 (1st Orchard)	Mango	Mango	15	Sorahie	42 ± 1.57	68 ± 1.33	18° 31 mm
						Gola	44 ± 0.67	70 ± 5.77	
	8.	Tandly wala 31.0368, 73.1379 (1st Orchard)	Glad	Glad	8	Sorahie	27 ± 1.15	37 ± 3.71	31° 184 mm
						Gola	29 ± 1.95	42 ± 1.15	
SINDH	1	Hyderabad 25.3960, 68.3578 (2nd Orchard)	Alfalfa	Wheat	10	Sorahie	30 ± 1.15	54 ± 3.05	28° 0 mm
						Gola	35 ± 1.50	56 ± 1.76	
	2	Larkana 27.5570, 68.2028 (1st Orchard)	Alfalfa	Berseem	15	Sorahie	31 ± 1.70	50 ± 5.77	27° 730 mm
						Gola	32 ± 0.47	58 ± 1.15	
KPK	1	Hazara 32.7962, 74.2840 (2nd Orchard)	Sorghum	Wheat	5	Sorahie	27 ± 0.88	30 ± 5.77	28° 100 mm
						Gola	29 ± 0.57	38 ± 1.15	
	2	Kohat 33.5889, 71.4429 (3rd Orchard)	Alfalfa	Wheat	20	Sorahie	35 ± 0.67	56 ± 3.05	28° 100 mm
						Gola	37 ± 0.58	59 ± 1.76	

The maximum disease severity and incidence was recorded 44% and 70% respectively in Punjab province, district Faisalabad where mango was cultivated as intercrop in guava orchard. The disease prevalence was high where mango and citrus were cultivated as intercrop in guava orchard. It was usually observed that disease severity and incidence were increased with the increase of age if the environmental conditions are the same.

Screening of guava intercrops to find their role in disease development

Samples were collected from eight different guava intercrops that were cultivated at different locations and processed. Different pathogens were isolated from the samples and identified on a morphological basis. The most frequently isolated pathogen was

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Colletotrichum species except Glad and Berseem samples. *Colletotrichum gloeosporioides* was isolated from the mango and citrus samples. The complete detail of frequency (%) of identified *Colletotrichum* species from different intercrops on a morphological basis were given below in table 4.4:

Table 4.4: Frequency (%) of different *Colletotrichum* species on intercrops

Sr no.	Intercrops	*Colletotrichum* Isolates		*Colletotrichum* Frequency (%)
		Genus	Species	
1	Sorghum	*Colletotrichum*	*graminicola*	73.3
2	Alfalfa	*Colletotrichum*	*trifolii*	60.0
3	Berseem	*Nill*	*Nill*	0.0
4	Maize	*Colletotrichum*	*Graminicola*	66.7
5	Wheat	*Colletotrichum*	*graminicola*	60.0
6	Mango	*Colletotrichum*	*gloeosporioides*	80.0
7	Glad	*Nill*	*Nill*	0.0
8	Citrus	*Colletotrichum*	*gloeosporioides*	86.7

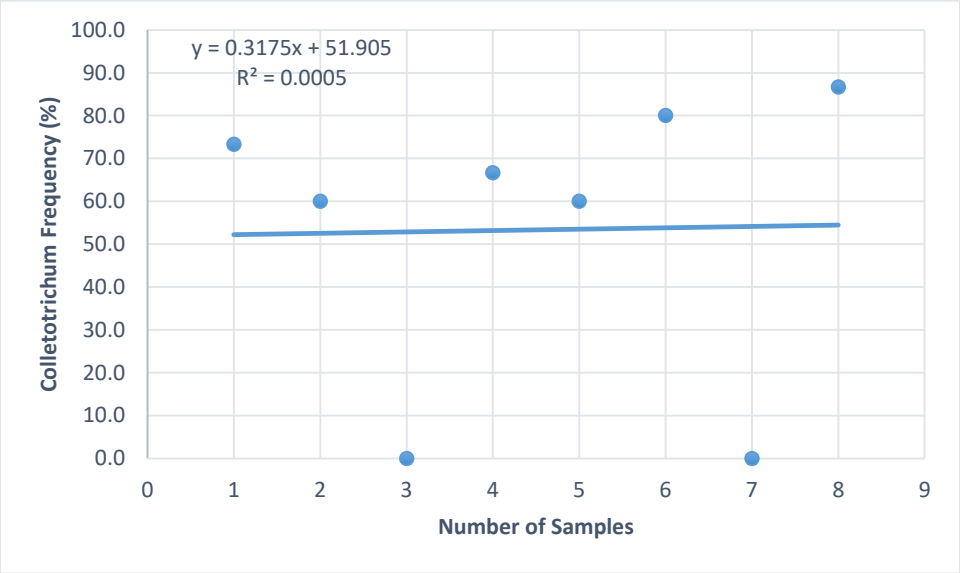

Figure 4.1: Frequency % of Colletotrichum species from eight different intercrops

Isolates from intercrops cultivated at different locations and Pathogenicity assays on Guava

The *Colletotrichum gloeosporioides* isolates, isolated from mango and citrus, were inoculated on guava plants. This experiment was conducted in control conditions and

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statistically managed by using the CRD design. The first data was recorded after four weeks of inoculation, and three more data were recorded in consecutive two-week intervals. All the isolates produced the disease symptoms on guava plants, and after ten weeks of inoculation, the highest disease severity, 40.67%, was found on those plants inoculated by Mcg2 isolate. According to data on disease severity, the mango isolates were more virulent than the citrus isolates. The complete detail of disease severity of all isolates after a different interval of time were given below in table 4.5:

Table 4.5: Disease severity (%) of *Colletotrichum gloeosporioides* isolated from mango and citrus on guava plants

Host	Isolates	Disease severity (%) ± Standard Error (S.E)			
		4th week	6th week	8th week	10th week
Mango	Mcg1	26.00±0.58	29.67±0.88	33.67±1.45	39.00±2.08
	Mcg2	28.00±1.15	31.33±1.20	35.00±1.15	40.67±1.76
Citrus	Ccg1	23.67±0.88	27.00±0.58	31.00±0.58	35.33±0.88
	Ccg2	22.67±1.08	26.00±0.68	29.67±0.98	35.67±1.45

Here Mcg1, Mcg2 represents the *Colletotrichum gloeosporioides* isolated from mango plants and Ccg1, Ccg2 represents the *Colletotrichum gloeosporioides* isolated from citrus plants

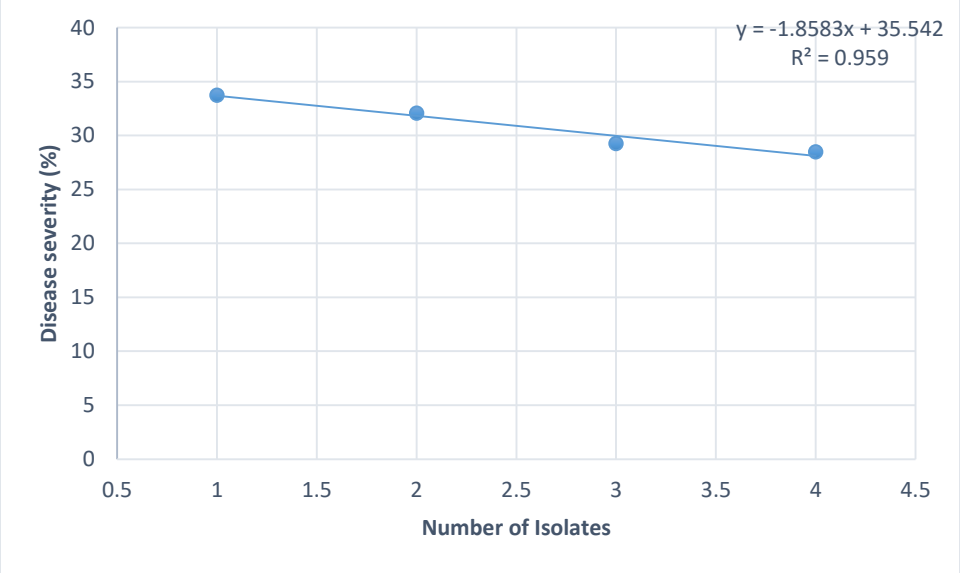

Figure 4.2: Ability of Colletotrichum gloeosporioides isolated from mango and citrus to cause disease in Guava

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Sequencing

After the confirmation of pathogenicity assay, these isolates were selected for sequencing. The genomic DNA of the fungal isolate was extracted using the method described by (Liu et al., 2012) with some modifications. Extracted DNA was quantified through Nano Drop 8000 spectrophotometer (Thermo fisher, USA), and working dilutions were made for polymerase chain reaction (PCR) analysis. For molecular identification, we used the primers in PCR analysis that were used by Iqra et al., 2021(A part of Ph.D. thesis and project research work on the same disease of Guava) and for the amplification of partial regions of ACT, CAL, TUB, ITS, and GAPDH genes to delimit the *Colletotrichum* species. The PCR products were resolved on agarose gel by electrophoresis at 80 volts. The gel was sliced at the brink of the required amplicon, and DNA of each sample was eluted through Gel purification Kit (Favor Prep) and cloned into TA cloning vector, pTZ57R/T (InsTAclone^{TM} PCR cloning kit), followed by direct sequencing through Eurofins Genomics DNA sequencing services, (USA). The sequences were trimmed through the Bio Edit tool (alignment editor) v. 7.2.6.1 to get high-quality (HQ) sequences. The HQ sequences were analyzed through the homology search tool, Blastn, which revealed 100% sequence similarity with isolate FMB-TnF.2-2(w)-Guv, (FMB 0137, NCBI accession no. MH618252, MN339477, MN401316, MN367317, MN308244) of *Colletotrichum gloeosporioides*.

Discussion

C. gloeosporioides is the broad host range pathogen that caused the dieback disease of guava in Pakistan and worldwide (Sharma and Kulshrestha, 2015). The literature was studied, which crops were cultivated as an intercrop in guava orchards worldwide and compared with data of intercrops in Pakistan and found the significant results, the intercrops were cultivated in Pakistan was different. Then the host range of *Colletotrichum* species was reviewed to determine whether the intercrops cultivated in Pakistan are a host of *Colletotrichum* species or not. All the intercrops were in the list of host range of *Colletotrichum* species except Glad and berseem.

Disease prevalence of intercropped guava orchards was recorded at different locations, and it was found high where mango and citrus were cultivated as an intercrop compared to other orchards. Eight different intercrops (Sorghum, Alfalfa, Berseem, Maize, Wheat, Mango, Glad, and Citrus) were observed commonly in guava orchards, these Intercrops were screened, the samples were collected from those intercrops and guava plants based on disease symptoms (Iftikhar et al., 2008; Bandgar et al., 2018; Mathur et al., 1997; Singh, 2008). Different *Colletotrichum* species were isolated from all the intercrops samples except Glad and berseem and *Colletotrichum gloeosporioides* isolated from mango and citrus samples, the frequency of each *Colletotrichum* species was more than 50%.

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After isolation, all the isolates were identified on a morphological basis. Ji and Guo, (1992) were identified the *Colletotrichum gloeosporioides* and *C. oleifera* by following the morphological examination and Baxter *et al*., (1985); Bose *et al*., (1973) were reported the shape and size of conidia of *Colletotrichum gloeosporioides.* Palo (1932), study the morphology of *Colletotrichum gloeosporioides* and observed the irregular spores, and Sattar and Malik, (1939) were observed the straight cylindrical, oval shape conidia and well-developed hyaline conidiophores.

The *Colletotrichum gloeosporioides* isolates from mango (Mcg1, Mcg2) and citrus (Ccg1, Ccg2) were selected for pathogenicity assay and inoculate the guava plants. After four weeks of inoculation, the first data was recorded, and for accurate results, three more readings were recorded with two weeks intervals. The results were significant; all the isolates caused the successful infection on guava plants, maximum disease severity of 40.67% was recorded after ten weeks, and the mango isolates were more aggressive than citrus isolates.

Araújo et al., 2016 was performed the Pathogenicity experiment and checked the aggressiveness of *Colletotrichum gloeosporioides*. Three different *Colletotrichum gloeosporioides* isolates Cg1 (Papaya), Cg2 (Guava), Cg3 (Mango), were selected and inoculated the three varieties of ornamental pepper in two different seasons and record the data of severity symptoms by grading scale and found the significant results, the isolates produced the symptoms on pepper plants and aggressiveness of each isolate was different. Dodd et al., (1991) was reported the anthracnose disease of mango which is caused by *C. gloeosporioides and its losses.* Similarly, Lima Filho et al. (2003) studied the cross-pathogenicity of *Colletotrichum* spp., testing *C. gloeosporioides* obtained from the cashew apple, passion fruit, mango, papaya, and *C. musae* (Berk & Curt.) von Arx. isolated from bananas in these same fruits. They observed that only the passion fruit isolates exhibited pathogenic specificity, while the others displayed cross-pathogenicity. In Belize, Fagan, (1980) was isolated the three strains of *C. gloeosporioides* such as: *cgm, cgc and cgp* and observed that *cgm* and *cgc* were nonpathogenic to citrus flowers. In Florida, Sonoda and Pelosi, (1988) were observed the slow and fast-growing strains of *C*. *gloeosporioides* which caused post-bloom fruit.

After the confirmation of pathogenicity assay, the sequencing results were proved, these *Colletotrichum gloeosporioides* isolates were similar that was isolated from intercrops and guava plants.

Conclusion

The effect of intercrops on guava dieback disease development was observed. Different *Colletotrichum* species were isolated from the intercrops except berseem and glad and *Colletotrichum gloeosporiodies* was isolated from mango and citrus intercrops. According to the pathogenicity and sequencing results the cross-infection of *Colletotrichum gloeosporiodies* among guava, mango and citrus were proved. The *Colletotrichum*

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gloeosporiodies isolates that were isolated from mango and citrus were produced the dieback disease symptoms on guava healthy plants. These results indicated that citrus and mango fruit crops are not suitable intercrops in guava orchards because these crops can play role in dieback disease development.

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